The Relationship Between Hydrogenase and Nitrogenase in *Azotobacter chroococcum*: Effect of Nitrogen Sources on Hydrogenase Activity

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The effects of different nitrogen sources on the H_2 -uptake hydrogenase activity were studied in batch-grown and carbon- or sulphate-limited chemostat cultures of *Azotobacter chroococcum*. Hydrogenase activity was significantly higher in N_2 -fixing than in NH_4^+ - or NO_3^- -dependent cultures under carbon limitation or in early-stationary phase batch growth but was not influenced by the nitrogen source in sulphate-limited cultures. Hydrogenase activity developed nearly two generation times later than nitrogenase activity during the transition from NH_4^+ - to N_2 -dependent growth in carbon-limited cultures. A possible explanation is that H_2 produced by nitrogenase induced hydrogenase synthesis. Carbon-limited N_2 -fixing cultures possessed higher hydrogenase activity than did sulphate- or O_2 -limited cultures. It appears that carbon substrates or metabolites act as primary repressors of hydrogenase activity and that secondary effectors, such as nitrogen sources or H_2 , act when such repression is removed.

INTRODUCTION

Wilson and his colleagues in the early 1940s established the presence of an H_2 -uptake hydrogenase in *Azotobacter vinelandii* which was most active when the bacteria were fixing N_2 (Wilson *et al.*, 1942; Lee *et al.*, 1942; Lee & Wilson, 1943; Green & Wilson, 1953). A reason for this relationship became apparent when it was established that the nitrogenase of *Azotobacter* species produced H_2 *in vivo*: Brotonegoro (1974) and Smith *et al.* (1976) showed that N_2 -fixing *Azotobacter chroococcum* evolved H_2 in the presence of CO and acetylene. Smith *et al.* (1976) offered the following explanation: CO inhibited N_2 or acetylene reduction by nitrogenase, thus diverting electrons to ATP-dependent H_2 production, and CO plus acetylene inhibited the uptake hydrogenase, thus preventing recycling of H_2 formed by nitrogenase. This established a rationale for the apparent association between hydrogenase and nitrogenase.

Several factors, largely recognized by Green & Wilson (1953), could influence hydrogenase activity and were not taken into account in the experiments by Wilson's group. These include: (1) enzyme activity might change during growth and different nitrogen sources might cause different growth rates; (2) excess O_2 may inhibit hydrogenase activity and the optimum O_2 concentration for maximum activity may vary with culture density; (3) pH might affect the rate of hydrogenase synthesis and hence activity in the assay, although the experiment of Lee & Wilson (1943) indicated that the nitrogen source rather than pH influenced activity; (4) the Knallgass reaction might not reflect the maximum H_2 -uptake

capacity of the enzyme and an assay involving fewer intermediate carriers was desirable. To overcome some of these criticisms, Green & Wilson (1953) measured hydrogenase activity in whole cells and in cell-free extracts with $K_3Fe(CN)_6$ or methylene blue as electron acceptors. Their observations supported the earlier conclusion that the hydrogenase was more active in N_2 -grown than in NH_4^+ -grown *Azotobacter*.

Walker & Yates (1978) showed that carbon-limited continuous cultures of *A. chroococcum* expressed a higher hydrogenase activity than did O_2 - or N_2 -limited cells. This implied that carbon substrates repressed or inhibited hydrogenase synthesis or activity. Repression of uptake hydrogenase activity by carbon substrates has also been observed in *Alcaligenes eutrophus* (Schlegel & Eberhardt, 1972) and *Rhizobium japonicum* (Maier *et al.*, 1978, 1979; Lim & Shanmugam, 1979). Other factors known to affect hydrogenase activity include O_2 and H_2 (Yates *et al.*, 1980). Since a reason for a relationship between hydrogenase and nitrogenase has been established and factors affecting hydrogenase activity which were not considered by the early investigators have been discovered, we have re-investigated the effects of nitrogen source on hydrogenase and nitrogenase in both batch and continuous cultures of *A. chroococcum*.

METHODS

Organism and growth. A non-gummy variant of *A. chroococcum* (NCIB 8003) was grown in batch or continuous cultures at 30 °C under air (Baker, 1968) in Burk's sucrose medium (Newton *et al.*, 1953) modified to contain 8.2 mM-mannitol instead of sucrose for carbon-limited chemostat cultures or 50 μM - Na_2SO_4 for sulphate-limited chemostat cultures. The nitrogen sources were either $NaNO_3$, ammonium acetate or NH_4Cl (18 mM) or N_2 . Carbon- and sulphate-limited chemostats contained 450 and 240 ml culture, respectively. Populations were shown to be carbon- or sulphate-limited by a proportional increase in culture turbidity on increasing the limiting nutrient concentration while retaining a positive dissolved oxygen tension in the cultures (O_2 was measured by a Uniprobe oxygen electrode and meter). Cultures were grown under N_2 -fixing conditions at pH 6.7 ± 0.1 , usually at a dilution rate of 0.1 h^{-1} , at pH 7.4 with $NaNO_3$ or ammonium acetate or at pH 6.4 with NH_4Cl . Steady-state turbidities ranged from 0.21 to 0.30 and 0.18 to 0.21 for N_2 -fixing sulphate- and carbon-limited cultures, respectively, and from 0.54 to 0.60 when the cultures were grown on fixed nitrogen regardless of the source or substrate limitation. Turbidities were measured in a 1 cm cell at 540 nm in an EEL colorimeter. Batch cultures were grown either in 100 ml medium in shaken conical flasks (250 ml) for 24 h or in air-sparged 20 l batches at 30 °C. Samples from the latter were harvested during growth.

Nitrogenase and hydrogenase assays. Nitrogenase activity was measured as the rate of acetylene reduction at 30 °C and interpolated for optimum O_2 concentration (Walker & Yates, 1978) using 5 ml samples from continuous cultures. Hydrogenase activity was determined by two procedures. (1) Activity was measured manometrically in washed cells suspended in phosphate buffer, pH 8.0, containing NaF (90 mM) and EDTA (2.4 mM) with methylene blue (28 mM) as the electron acceptor. EDTA and NaF were added routinely because in pilot experiments with *A. vinelandii*, where hydrogenase was assessed by decolorizing methylene blue, EDTA and NaF stimulated the reaction rate several-fold (F. Simpson & J. R. Postgate, unpublished observation). EDTA and NaF stimulated hydrogenase activity in manometric assays, where the methylene blue concentration was saturating, only when the cells produced a high concentration of extracellular polysaccharide (C. C. Walker, unpublished observation); nevertheless, as a precaution, they were added to all assays involving methylene blue as an electron acceptor. This assay and that for nitrogenase were assumed to be measures of the hydrogenase and nitrogenase contents of the cells. (2) H_2 uptake with O_2 as the terminal electron acceptor was measured by injecting H^3H , produced from sodium borohydride, either into the chemostat in air or into 5 ml samples under H_2 (70 %) and optimum O_2 concentration (10 to 15 % depending on the population density and shaking rate) with argon as the normalizing gas. H^3HO was estimated in a Nuclear Enterprises scintillation counter with NEN 250 as the scintillant.

Assay of nitrogenase peptides. Nitrogenase peptides in whole cells were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (Robson, 1979) and estimated visually after staining with Coomassie blue.

RESULTS

Hydrogenase activity in batch cultures

The specific activity of the H_2 -uptake hydrogenase in batch cultures grown in shake flasks, harvested late in growth, was significantly higher when nitrogenase was derepressed

Table 1. *Effect of different nitrogen sources on the uptake hydrogenase activity of batch cultures of Azotobacter*

Results in columns 1 (present investigation) and 4 (from Green & Wilson, 1953) were obtained by measuring methylene blue-dependent H_2 uptake manometrically; those in columns 2 and 3 (from Lee & Wilson, 1943) were obtained by measuring O_2 -dependent H_2 uptake manometrically. In each case the results are expressed as a percentage of the activity of hydrogenase in an N_2 -fixing culture since the specific activities were not all expressed in the same units.

Nitrogen source	Relative hydrogenase activity			
	<i>A. chroococcum</i>		<i>A. vinelandii</i>	
	(1)	(2)	(3)	(4)
N_2	100	100	100	100
NH_4^+	59	31.2	28.1	56
NO_3^-	48.5	81	60.8

Fig. 1. Uptake hydrogenase activity of N_2 -fixing *A. chroococcum* in batch culture. Twenty cultures (100 ml each in 250 ml conical flasks; 1% inoculum) were grown on a rotary shaker at 30 °C. They were harvested (maximum of four flasks, minimum of two, depending on the cell density) after the indicated time intervals by centrifugation at 30000 g for 10 min at 5 °C, resuspended in phosphate buffer, pH 8.0 plus EDTA and NaF and then assayed for hydrogenase manometrically with methylene blue as the electron acceptor (see Methods). The culture turbidity was measured at 540 nm. ■, Hydrogenase activity; ▲, A_{540} .

(Table 1). These results are similar to those of Lee & Wilson (1943) (Table 1, columns 2 and 3) and Green & Wilson (1953) (Table 1, column 4). The NO_3^- -grown cells had grown in continuous culture for 86 to 87 generations before adapting to NO_3^- and repressing nitrogenase. During this time, batch cultures inoculated from the continuous culture possessed hydrogenase activities as high as those in N_2 -fixing cells. After adaptation, the hydrogenase activity decreased to 48.5% (Table 1). Green & Wilson (1953) showed that hydrogenase activity diminished as N_2 -fixing cultures aged, but the reverse occurred in NH_4^+ -grown cells. This increase in NH_4^+ -grown cells coincided with the disappearance of NH_4^+ from the supernatant, again consistent with a relationship between hydrogenase and nitrogenase.

However, hydrogenase activities measured with methylene blue varied widely among replicate batch cultures, ranging between 473 and 967 $\mu\text{mol } H_2 \text{ absorbed (mg protein)}^{-1} \text{ min}^{-1}$ for N_2 -grown cells, between 28 and 408 for NH_4^+ -repressed cells and between 80 and 606 for NO_3^- -repressed cells. Figure 1 indicates why this was so: consistent with Green & Wilson's (1953) findings, hydrogenase activity changed markedly during batch growth, increasing during cell multiplication and declining in the stationary phase. A similar

Fig. 2. Effects of nitrogen source on uptake hydrogenase and nitrogenase activities in carbon-limited *A. chroococcum*. Hydrogenase was assayed manometrically with methylene blue and nitrogenase was assayed by the rate of acetylene reduction at optimum O_2 concentration (see Methods). At time zero, the ammonium acetate-dependent culture ($D = 0.1 \text{ h}^{-1}$, pH 7.5) was shifted to nitrogen-free medium; the first arrow indicates the shift back to ammonium acetate and the second arrow the subsequent return to N_2 -dependent growth. The culture was carbon-limited because excess O_2 was detected in the medium. ■, Hydrogenase activity; ●, nitrogenase activity.

pattern was observed whether the pH was controlled (pH 7.3) or uncontrolled (changing from pH 7.4 to 6.1 during growth).

Hydrogenase and nitrogenase activity in continuous cultures

The H_2 -uptake activity of a given carbon- or sulphate-limited culture did not change significantly in the steady state. Different activities were observed in different cultures under similar nutrient limitations. Whether this was due to phenotypic variation among the populations or different physical parameters in the culture vessels is not known.

Carbon-limitation. Both the nitrogenase and the H_2 -uptake hydrogenase activities increased significantly during the transition from ammonium acetate- to N_2 -dependent growth in carbon-limited cultures; this increase was reversed during the transition back to NH_4^+ -dependence but occurred again on transferring back to N_2 utilization (Fig. 2). This pattern was also obtained with NH_4Cl as the source of fixed nitrogen. The increase in hydrogenase activity commenced several hours later than the increase in nitrogenase activity, implying that the two enzymes were not derepressed co-ordinately. Specific activities of hydrogenase in carbon-limited, N_2 -fixing populations were slightly less than the lowest activity observed in early-stationary phase batch cultures.

Sulphate-limitation. Uptake hydrogenase activity in N_2 -grown sulphate-limited cultures was generally lower than that in carbon-limited cultures: ranging from 30 to 100, compared with 60 to 275 $\mu\text{mol } H_2 \text{ absorbed (mg protein)}^{-1} \text{ min}^{-1}$ in the latter cultures. During the transition from N_2 - to NH_4^+ -supported growth there was a rapid decline in nitrogenase activity, faster than the washout time of the N_2 -fixing culture or the disappearance of nitrogenase peptides from the extracts. Hydrogenase activity, on the other hand, declined by 25 % in 7 h but returned to the same level as that in N_2 -fixing cells at the steady state in NH_4^+ -grown cultures (Fig. 3). A similar transient decline in uptake hydrogenase activity was observed during the reverse transition from NH_4^+ - to N_2 -dependent growth. Measurements of O_2 -dependent H^3H oxidation confirmed that the hydrogenase activity of sulphate-limited cells was independent of the nitrogen source.

Effect of dilution rate. The uptake hydrogenase activity of an N_2 -dependent, sulphate-limited population declined by 22 % on changing the dilution rate from 0.05 to 0.1 h^{-1} and

Fig. 3. Uptake hydrogenase and nitrogenase activity in sulphate-limited *A. chroococcum* during transition from N_2 - to NH_4^+ -dependent growth. Hydrogenase and nitrogenase were measured as described in the legend to Fig. 2. The arrow indicates the steady state values under NH_4^+ -grown conditions. ■, Hydrogenase activity; ●, nitrogenase activity.

Table 2. Effect of dilution rate on nitrogenase and hydrogenase activities in N_2 -fixing sulphate-limited *A. chroococcum*

Nitrogenase activity was determined in samples from continuous cultures in steady state as described in Methods; results are expressed as nmol acetylene reduced (mg protein)⁻¹ min⁻¹. Hydrogenase activity was measured manometrically with methylene blue as the electron acceptor; results are expressed as nmol H_2 absorbed (mg protein)⁻¹ min⁻¹.

Dilution rate (h ⁻¹)	Hydrogenase activities	Nitrogenase activity
0.05	139 140	NT
0.10	90 115 129	12.5
0.15	99 95	22.2
0.20	108 117 118	44.0

NT, Not tested.

thereafter remained approximately the same at dilution rates of 0.15 and 0.2 h⁻¹. On the other hand, nitrogenase activity increased fourfold on increasing the dilution rate from 0.1 to 0.2 h⁻¹ (Table 2). The percentage of nitrogenase peptides in the total cell extract also increased over this range of dilution rates. Hence, as in carbon-limited populations (Fig. 2), nitrogenase and uptake hydrogenase activities were not co-ordinately regulated in sulphate-limited *A. chroococcum*. Effects of dilution rate on carbon-limited populations were not studied.

Effect of pH. The transition from N_2 - to ammonium acetate-dependent growth in sulphate-limited cultures caused an increase in pH from 6.7 to 7.5. However, the hydrogenase activity did not change during the transition between nitrogen sources when the pH was kept at pH 6.9 by manual additions of HCl. Hydrogenase activity is thus not permanently affected by pH changes of the culture medium between 6.7 and 7.4 in sulphate-limited cells. Furthermore, the pH change (7.5 to 7.0) accompanying the transition from NH_4^+ - to N_2 -dependence in carbon-limited cultures preceded the onset of the increase

in hydrogenase activities by almost two generation times. This again implies that this change in external pH did not affect hydrogenase activity. Lee & Wilson (1943) also found that the pH of the culture within this range had no effect on the specific activity of hydrogenase in extracts from *Azotobacter*.

Effects of H_2 . Exposure of an ammonium acetate-supported carbon-limited culture to 5% H_2 in the atmosphere for 12 h doubled hydrogenase activity. No effect was observed on the uptake hydrogenase activity of a similarly treated ammonium acetate-supported sulphate-limited culture. The effects of H_2 on hydrogenase activity in N_2 -dependent chemostat cultures were not studied.

DISCUSSION

The use of continuous culture techniques in this study has clearly overcome some of the disadvantages encountered in batch cultures of *A. chroococcum*, in which hydrogenase activities varied considerably between batches of the same age. Steady-state hydrogenase activities were consistent within a given chemostat run. For the purpose of this discussion we assume that the measured activity of hydrogenase related to the hydrogenase content and synthesis of active enzyme.

In carbon-limited chemostat cultures, in the batch cultures used by Wilson and his colleagues and in our stationary phase batch cultures, the uptake hydrogenase activity was greater under N_2 -fixing conditions than when nitrogenase synthesis was repressed by the source of fixed nitrogen. However, even in carbon-limited conditions, it is unlikely that the syntheses of nitrogenase and hydrogenase are controlled by the same regulator for two reasons: (i) NH_4^+ represses hydrogenase synthesis 2- to 7-fold whereas it represses nitrogenase by more than 10^4 -fold; (ii) nitrogenase synthesis precedes hydrogenase synthesis by approximately 12 h (Fig. 2). The results in Fig. 2 suggest a more likely possibility: that hydrogen, a normal product of nitrogenase action, induces hydrogenase synthesis. The effect of exogenous 5% H_2 in augmenting the hydrogenase activity of NH_4^+ -dependent carbon-limited populations supports this view. The effect of exogenous H_2 was small compared with the effect of changing the nitrogen source from NH_4^+ to N_2 , but the position is complicated by an inhibitory effect of excess exogenous H_2 : increasing the H_2 in the gas phase above 5% inhibited batch growth of *A. chroococcum* (A. van der Werf & M. G. Yates, unpublished results) and decreased hydrogenase activity (Lee & Wilson, 1943). It is therefore plausible that fixed nitrogen sources have no direct influence on hydrogenase synthesis but that derepression of nitrogenase leads to H_2 -induced synthesis of extra hydrogenase.

The specific activities of hydrogenase in carbon-limited N_2 -fixing chemostat cultures were generally higher than under other nutrient limitations and resembled those of batch cultures at the end of their growth curve, when carbon sources may be limiting. These observations support the view (Walker & Yates, 1978) that hydrogenase synthesis in *A. chroococcum* is primarily repressed by carbon metabolites and that this process overrides the effects of nitrogen source discussed above.

Two other features of hydrogenase synthesis require explanation. Firstly, the decrease in hydrogenase activity upon increasing the dilution rate of sulphate-limited N_2 -fixing cells from 0.05 to 0.1 h^{-1} probably arose because the faster growth allowed a higher steady-state level of a carbon metabolite repressor than that necessary for maximum derepression. Secondly, nitrogenase activity declined faster than hydrogenase activity in carbon-limited cells during the transition from N_2 - to NH_4^+ -supported growth: nitrogenase activity declined to zero within 6 h, faster than both the washout time and the rate of disappearance of nitrogenase peptides from the N_2 -fixing population, whereas hydrogenase activity declined at the same rate as the washout of N_2 -fixing cells. This is consistent with other reports that NH_4^+ can specifically inhibit nitrogenase activity in *Azotobacter* species (L'vov *et al.*, 1971; Drozd *et al.*, 1972; Kleiner, 1975).

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